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Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein gene transcription

(peptide hormone receptors/transmembrane signaling/growth hormone/cytokines)

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ABSTRACT **The biological activities of long and short forms of the prolactin receptor have been compared. These two receptors expressed in mammalian cells were shown to bind prolactin with equal high affinity. The ability of these different forms to transduce the hormonal message was estimated by their capacity to stimulate transcription by using the promoter of a milk protein gene fused to the chloramphenicol acetyltransferase (CAT) coding sequence. Experiments were performed in serum-free conditions to avoid the effect of lactogenic factors present in serum. An ≈17-fold induction of CAT activity was obtained in the presence of prolactin when the long form of the prolactin receptor was expressed, whereas no induction was observed when the short form was expressed. The present results clearly establish that only the long form of the prolactin receptor is involved in milk protein gene transcription.**

The anterior pituitary hormone prolactin (PRL) first interacts with specific receptors located in cell membranes of many target tissues (1). The best characterized biological action of PRL involves the activation of milk protein gene expression by increasing both gene transcription and mRNA stabilization (2). The intracellular mechanisms by which PRL induces these different effects following binding to its receptor remain unknown. A significant advance has been the cloning of cDNAs of PRL receptors (PRL-Rs) in several organs and species (3–7), allowing determination of the primary structure of several types of PRL-R molecules. These receptors differ by the size of their intracellular domains, postulated to be the region of the receptor responsible for signal transduction. PRL and growth hormone receptors have regions of significant homology localized in both extracellular and intracellular domains, suggesting that these receptors form a family of single membrane-spanning receptors. It has been recently suggested (8) that this family could be extended and now includes the receptors for erythropoietin (9), interleukin 2 (IL-2) (10), IL-3 (11), IL-4 (12), IL-6 (13), IL-7 (14), granulocyte–macrophage colony-stimulating factor (15), and granulocyte colony-stimulating factor (16).

The first type of PRL-R originally identified in rat liver has a short (57 amino acids) intracellular domain, and the second type, identified originally in rabbit mammary gland, has a longer one (358 amino acids). These two forms may coexist in the same tissue, at least in the rat (7). The highest degree of homology in the cytoplasmic domain between PRL and growth hormone receptors is located in the first 29 intracellular amino acids common to both short and long forms of the PRL-R. This restricted region could be crucial for signal transduction. Also in the same family of receptors, several reports indicate that short forms of interleukin receptors [IL-6 receptor (17)] could be fully biologically active. The aim of the present study was to compare the potency of the two forms of PRL-R in stimulating milk protein gene transcription. We recently demonstrated by cotransfection experiments in CHO cells that the long form of the rabbit mammary gland PRL-R is able to stimulate a PRL-responsive gene (β-lactoglobulin) in the presence of PRL. The effect obtained was moderate (5-fold induction), probably because an increase of the basal transcriptional activity occurs in cells transfected with the PRL-R cDNA, even in the absence of PRL. This could be due to lactogenic factors present in the serum of the culture medium. This renders the assay difficult for accurate comparison of the potency of different forms or mutants of the PRL-R.

In the present study, the use of serum-free culture conditions, which greatly increase the amplitude of the response of this functional assay, permits the demonstration that the short form of PRL-R is unable to stimulate β-lactoglobulin gene transcription, emphasizing that the two receptor forms mediate different biological effects.

MATERIALS AND METHODS

Transient Expression Procedures and Chloramphenicol Acetyltransferase (CAT) Assay. CHO K1 cells were grown in complete medium [Ham's F-12/10% fetal calf serum (FCS)] until 50% confluence on 60-mm culture dish. Three hours before transfection, cells were washed twice with Earle's modified Eagle's medium (EMEM) and serum-free medium was added. This medium is derived from GC_3 medium described by Gasser *et al.* (18) and is a 1:1 mixture of EMEM and Ham's F-12 supplemented with transferrin (10 μg/ml), insulin (80 milliunits/ml), glutamine (2.5 nM), and nonessential amino acids (Flow Laboratories). Cells were transfected by the calcium phosphate precipitation procedure (19) with 3 μg of pCH110 (β-galactosidase expression vector from Pharmacia), 1.5 μg of either pBJ23 [the reporter gene containing 4 kilobases of the β-lactoglobulin promoter followed by the bacterial CAT coding sequence (20)] or plasmid controls: pTKCAT (21), pActinCAT (22), and 3 μg of either pER_11 [pECE-PRL-R short form (pECE/F3 in ref. 3)] or pER_23 [pECE-PRL-R long form (20)]. In pER_11 and pER_23, the cDNAs were placed under transcriptional control of simian virus 40 early promoter. After the glycerol shock, fresh GC_3 medium was added with or without 18 nM ovine PRL (oPRL). Forty-eight hours after transfection, GC_3 medium was replaced by medium with or without 18 nM oPRL. The next day, cells were scraped and lysed by repeated freeze–thaw cycles. After 10 min of centrifugation at 15,000 × *g*, the

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Abbreviations: PRL, prolactin; oPRL, ovine PRL; PRL-R, PRL receptor; IL, interleukin; CAT, chloramphenicol acetyltransferase; FCS, fetal calf serum.

supernatant was collected. Aliquots were normalized for β-galactosidase activity (23) and CAT assays were performed for 90 min as described (24) using 0.1 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol (Amersham). Acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography and quantitated by liquid-scintillation counting. Values are expressed as percentage chloramphenicol conversion and as -fold induction calculated from basal level activity.

Stable Expression Procedures and Scatchard Analysis. Stable CHO cell lines were established by cotransfection of 8 μg of $pER_1 1$ or $pER_2 3$ with 2 μg of pSV2neo using the calcium phosphate medium (19). Cells were maintained in complete Ham's F-12 medium with 10% FCS and selection was made with 300 μg of G418 per ml (Sigma). After isolation of the transfectant clones and amplification, cells were scraped with 1 ml of cold 25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/2 mM EDTA, and lysed in Eppendorf tubes by three freeze–thaw cycles. Membranes were prepared by centrifugation (10,000 × *g*) for 5 min and the pellet was resuspended in 25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. CHO cell membranes (100 μg) were incubated in the presence of ^{125}I-labeled oPRL. Binding studies and Scatchard analysis were performed as described (25). oPRL (NIADDK oPRL-16; 30.5 international units/mg) was kindly provided by the National Hormone and Pituitary Agency (Baltimore).

Western Blot. COS-7 cells were transfected with 8 μg of $pER_1 1$ or $pER_2 3$. Membrane preparations were carried out as described for the stable assays. For immunoblot analysis, 100 μg of suspension was run on a SDS/12% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was saturated overnight in 10 mM NaH_2PO_4/150 mM NaCl/0.05% Tween 20 and incubated with a goat anti-PRL-R antiserum diluted 1:100 in the same buffer. The receptor was revealed with a second antibody conjugated to alkaline phosphatase.

Statistics. Student's *t* test was used to analyze differences between control and PRL-containing cultures.

RESULTS

Serum-Free Medium in Transfection Experiments with PRL-R cDNA. We have recently shown that cotransfection of CHO cells with the rabbit PRL-R cDNA along with a fusion gene containing the promoter of the ovine β-lactoglobulin followed by CAT coding sequence (pBJ23) results in a clearly inducible CAT activity by the lactogenic hormones (20).

In the following experiments, we modified the cell culture conditions and used serum-free medium for CHO cells (18). As shown in Fig. 1, cells plated in the absence of FCS grew as well as those grown in a medium containing 10% FCS, with a doubling time in the range of 13–20 hr. These cells proliferated over a period of at least 5 days, and, after confluency was reached, they readily accepted transfection by calcium phosphate precipitation followed by a glycerol shock (19).

The use of serum-free conditions in the cotransfection experiments described above resulted in a very low basal CAT activity when the cells were cultured in the absence of PRL. Fig. 2 shows a comparative experiment: when cells were cultured in the presence of FCS, the cotransfection procedure resulted in a spontaneous increase of the CAT activity corresponding to 4.0% of chloramphenicol conversion, whereas in the absence of serum, only 0.7% conversion was observed. The addition of PRL induced a significant increase of the CAT activity under both conditions, but the amplitude of the response was much higher (16.5-fold induction) when serum-free medium was used. Also the cotransfection of plasmids containing different control promoters [pTKCAT (21) and pActinCAT (22)] with the expression plasmid of PRL-R cDNA did not show any induction of the CAT activity by PRL. As expected, PRL did not induce CAT activity in cells transfected by pBJ23 alone. This modification of the technique greatly improves the sensitivity of the functional test of PRL-R making it suitable for a more precise comparison of different forms of receptors.

Comparison of Functionality of Short and Long Forms of the PRL-R. Two cDNAs of PRL-R (rat short form and rabbit long form) were introduced in the pECE expression vector and transfected into CHO cells. Scatchard analysis (Fig. 3) demonstrated that the expressed receptors bind ^{125}I-labeled oPRL with high affinities ($K_a = 5.7 \times 10^9\ M^{-1}$ for the rat short form and $K_a = 2.8 \times 10^9\ M^{-1}$ for the rabbit long form), similar to values reported for PRL-R in rat liver (26, 27) and rabbit mammary gland, respectively (25). These results clearly indicate that in CHO cells these cDNAs direct the synthesis of PRL-Rs that have the same characteristics as native receptors. These proteins were also detected by Western blot analysis of membranes from transfected COS-7 cells (Fig. 4). These experiments revealed a major specific band migrating at $M_r \approx 88{,}000$ for the cDNA of the long form of PRL-R and a band at $M_r \approx 40{,}000$ for the cDNA of the short form of PRL-R, corresponding to the expected molecular weights of the two forms (Fig. 4). Similar experiments conducted with CHO cells failed to clearly reveal PRL-R in Western blots due

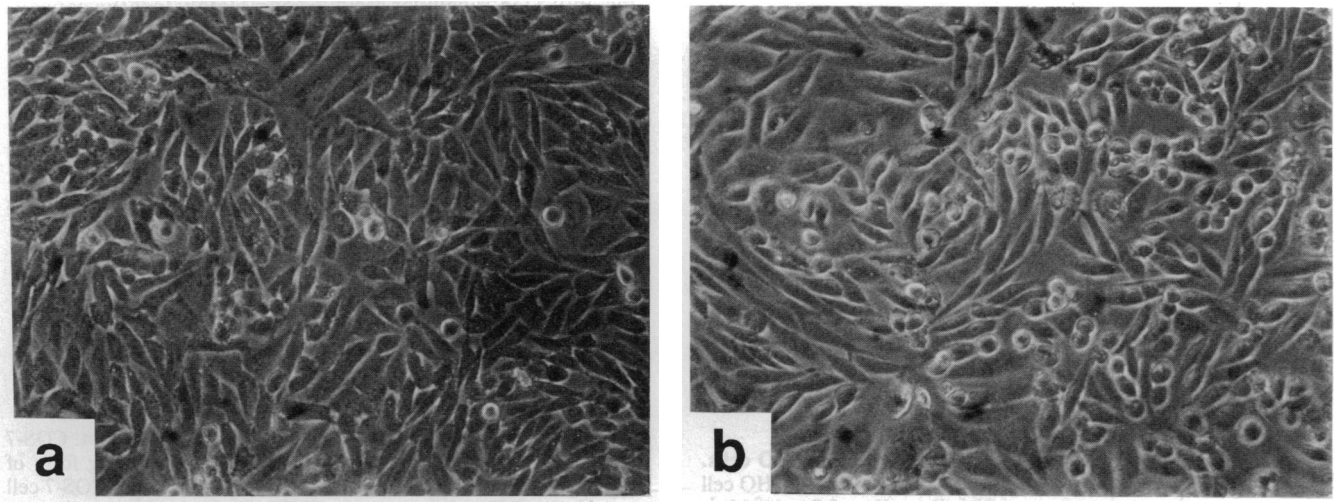

FIG. 1. Phase-contrast photomicrograph of CHO cells 3 days after plating. (*a*) Cells are growing in a medium containing 10% FCS. (*b*) Cells are growing in serum-free culture medium. (×180.)

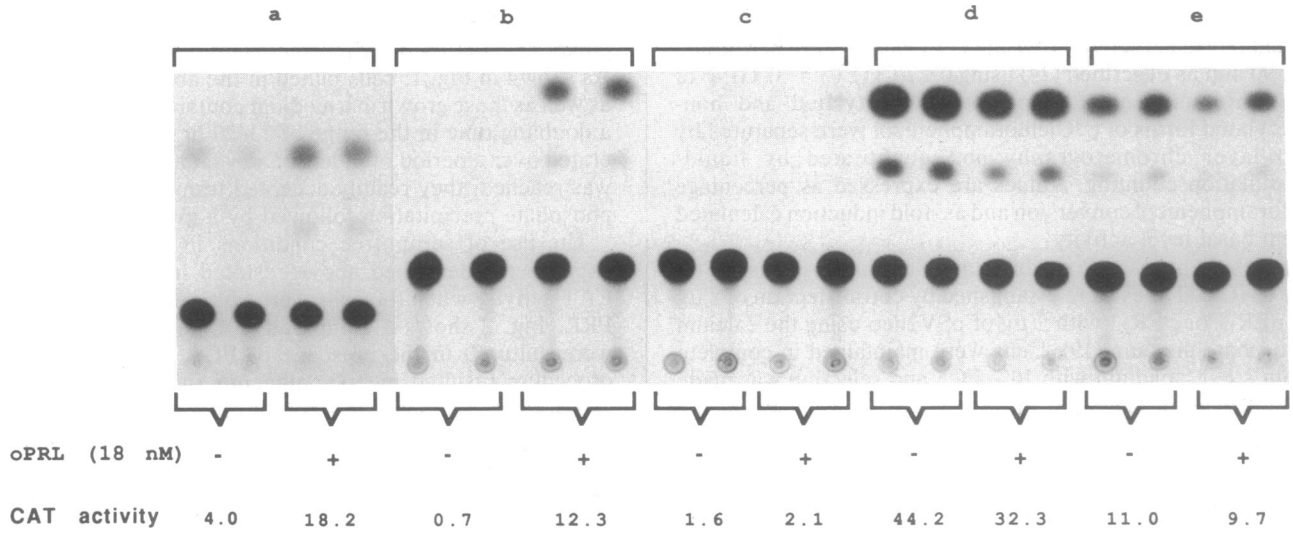

FIG. 2. Functional expression of PRL-R cDNA in transiently transfected cells. Transfection experiments were performed on cells cultured in medium containing serum (*a*) or in serum-free medium (*b–e*). Cells were untreated (−) or treated (+) with 18 nM oPRL and CAT activity is expressed as percent chloramphenicol conversion. Cells were transfected either with the reporter gene (pBJ23) and the cDNA of the long form of PRL-R (*a* and *b*), with pBJ23 alone (*c*), with pTKCAT and the cDNA of the long form of PRL-R (*d*), or with pActinCAT and the cDNA of the long form of PRL-R (*e*). For the pTKCAT plasmid, the CAT assay was performed at a 1:10 dilution.

to a lower level of expression in this cell line. We then analyzed the functionality of these two types of receptors by using the biological test described above. As shown in Fig. 5*a*, the cotransfection of the short form of PRL-R cDNA with the reporter gene resulted in the absence of CAT induction in the presence of PRL (1.3-fold induction), whereas the long form of PRL-R cDNA led to a marked increase of the CAT activity (16.1-fold induction). In six separate experiments (Fig. 5*b*), the basal ratio of chloramphenicol conversion (i.e., in the absence of PRL in medium) was 0.8% ± 0.3% (SEM) and 1.7% ± 0.4% (SEM) when the long form and the short form were expressed, respectively. In the presence of the optimal concentration of PRL (18 nM), CAT activity increased significantly to 13.8% ± 3.5% (17-fold induction; $P < 0.01$) with the long form, whereas no significant induction could be detected with the short form of PRL-R (1.1% ± 0.3%). We further analyzed the effect of increasing concentrations of PRL on cells transfected with the two types of PRL-R cDNAs. As shown (20), a dose–response of CAT activity was obtained with increasing concentrations of PRL in culture medium of cells transfected with the cDNA of the long form of PRL-R. A similar study conducted with the cDNA of the short form of PRL-R revealed that variations of the PRL concentration in the medium had no effect (data not shown), suggesting that the lack of response with the short form is not a concentration-related phenomenon.

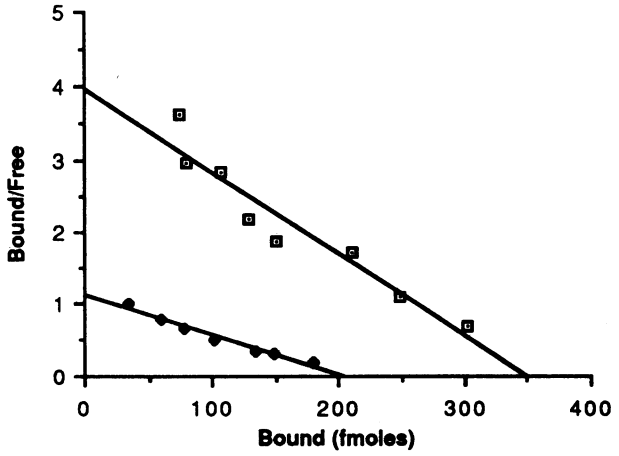

FIG. 3. Expression of the PRL-R cDNA clones in CHO cells. Scatchard plot of PRL binding data in a stably transfected CHO cell line expressing the rat short form of PRL-R (⊡, $K_a = 5.7 \times 10^9\ M^{-1}$; $n = 350$ fmol/mg) and the rabbit long form of PRL-R (◆, $K_a = 2.8 \times 10^9\ M^{-1}$; $n = 201$ fmol/mg).

DISCUSSION

PRL is recognized by at least two types of PRL-Rs recently cloned from rat liver (3) and rabbit mammary gland (5). These two forms differ by the sequence encoding the intracellular domains of each receptor, which are postulated to be responsible for the transduction of hormone signals inside target cells. The present results clearly establish that although these two forms are able to bind PRL with equal affinity, they are clearly involved in different biological functions. Only the long form of PRL-R is able to stimulate the transcription of milk protein genes, while the 57 amino acids in the intracellular domain of the short form of PRL-R are insufficient to transduce this effect. This is reminiscent of what has been shown for IL-2 receptor, in which a truncated form containing only 27 intracellular residues completely lost the capacity

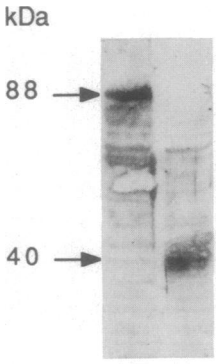

FIG. 4. Identification of PRL receptors by Western blot. COS-7 cells were transfected either with the cDNA of the long form of PRL-R or with the short form. Proteins from transfected COS-7 cell membranes (100 μg) were run in SDS/polyacrylamide gel. The gel was then electrotransferred onto nitrocellulose and the proteins were identified by an anti PRL-R antiserum.

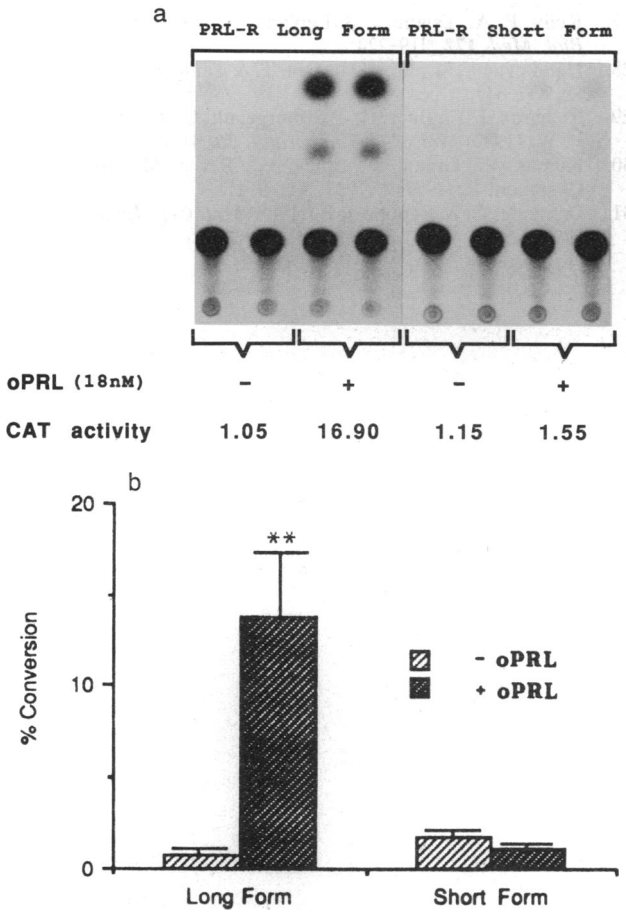

FIG. 5. Comparison of the biological activities of the long form and the short form of PRL-Rs. (*a*) CHO K1 cells were cotransfected with the PRL-R cDNAs and the reporter gene pBJ23. oPRL (18 nM) was added to (+) or omitted from (−) the serum-free medium during the experiment. The CAT assay was performed on extracts normalized for β-galactosidase activity and CAT activity is expressed as percent chloramphenicol conversion. (*b*) Each value represents the mean ± SEM of six separate experiments. **, $P < 0.01$.

to transduce hormonal signal (28). The biological effects that are specifically associated with the short form of PRL-R remain to be identified. One intriguing observation is that the short PRL-R represents the major form in the rat mammary gland, since only 30% of the total receptor population is the long form (G. Jahn, M.E., P.A.K., and J.D., personal communication). This polymorphism of receptor structure appears to be a general phenomenon observed in the newly identified superfamily of receptors, which includes those of PRL, growth hormone, and several cytokines (8), in that the size of the intracellular domains varies, and in fact soluble binding proteins are sometimes expressed. The members of this family are all involved in mitogenic effects. Prolactin is well known to induce cell proliferation in different cell types and particularly in the lymphocyte Nb_2 cell line model (29). The development of a specific functional assay to analyze the mitogenic capacity of different forms of PRL-Rs will be necessary to better understand the role of the short PRL-R. The structural arrangement of this short form is reminiscent of several receptors that act primarily as transporters (transferrin, low density lipoprotein, and insulin-like growth factor II/mannose 6-phosphate receptors). In fact, PRL has been detected in numerous biological fluids and particularly in milk where, in addition to the hormone, a soluble form of PRL-R has been recently identified (J.D., unpublished results).

The results presented in this paper illustrate that the approach using cotransfection of a receptor cDNA and a target reporter gene, widely used for steroid receptor studies (30), is also valid for peptide hormone receptors, even if they only moderately activate gene transcription (31). The improvement of the sensitivity and accuracy of the assay developed in the present work by using serum-free conditions for culture of CHO cells was the determining factor for the reduction of basal CAT activity, probably due to the presence of lactogenic factors in the serum, capable of interacting with the newly expressed PRL-R.

This biological model provides a valuable tool for studying the different steps, after binding of PRL to its receptor, leading to the activation of gene transcription. Site-directed mutagenesis and construction of truncated forms of PRL-R cDNA will allow localization of functional critical domains in PRL-R molecules.

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